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What is Hot and New in Basic Science in Liver Transplantation in 2018? Report of the Basic Science Committee of the International Liver Transplant Society.

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Abbreviations:

DCD = donation after circulatory death

DDLT = deceased donor liver transplantation

HCC = hepatocellular carcinoma

IRI = ischemia reperfusion injury

LDLT = living donor liver transplantation

NMP = normothermic machine perfusion

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Introduction

The 2018 Joint International Congress of the International Liver Transplant Society (ILTS), European Liver and Intestine Transplant Association (ELITA), and Liver Intensive Care Group of Europe (LICAGE) meeting was held in Lisbon, Portugal in May 2018. It was attended by 1,200 delegates from 58 countries (Figure 1). Ten percent of the 759 abstracts were Basic Science or Translational Research and were published in Transplantation.[1] In addition, all oral presentations are available to the ILTS members at the ILTS website.[2]

The ILTS Basic Science Committee (BSC) has selected 33 as representing the most interesting and innovative research covering the key areas of ischemia reperfusion injury/organ preservation, organ bioengineering, biliary injury/regeneration, transplant immunobiology, biomarkers, and acute kidney injury after liver transplant.

Ischemia-Reperfusion Injury (IRI) and Organ Preservation

Perfused liver preservation continues to be an extensively investigated area, with a focus on the role of normothermic machine perfusion (NMP) for the preservation of marginal and DCD (donation after circulatory death) liver grafts. Boteon et al.[3] assessed 46 discarded human liver grafts using lactate clearance as marker of viability. The authors compared NMP alone with a combination of hypothermic perfusion followed by normothermic ex vivo perfusion. They found that 52% of all discarded grafts were considered viable during NMP, as determined by lactate clearance, with viability being better for livers treated with combined cold plus warm ex vivo perfusion (71%) vs NMP alone (41%).[3]

Steatotic liver grafts remain to be the limiting factor for graft utilization and poor graft function post liver transplantation, as previously demonstrated.[4] A novel 'defatting' technique during 24hr NMP was also investigated by Boteon et al.[5] in 6 discarded human steatotic grafts. Using a specific defatting cocktail in the perfusate the authors were able to decrease

macrosteatosis by 18% as determined by histology, and at the same time increase perfusate triglyceride and cholesterol levels. Bile production was also improved by the treatment.

RNA interference (RNAi) is a natural process of post transcriptional gene regulation that was the subject of the Nobel Prize in Medicine in 2006.[6] Moore et al.[7] studied the utilization of gene silencing during NMP preservation. The authors used siRNA to silence the anti-apoptotic gene p53 associated to nanoparticles in both an *in vivo* rat model of liver clamping and a blood reperfusion model of DCD rat liver after machine perfusion preservation using siRNA+nanoparticles added to the perfusate. They showed that markers of liver inflammation and lipid peroxidation were inhibited and siRNA was taken up by the liver during NMP.[7]

Small for size syndrome is a relatively common problem after living donor liver transplant (LDLT). It has been shown previously that small-for-size liver grafts (SFSG) are associated with suppressed mitochondrial biogenesis and increased TGF-beta. Liu et al.,[8] using a rodent liver transplant model, showed that mitochondrial biogenesis occurred in a 50% size graft, however, there was significant evidence that mitochondrial biogenesis was blunted and there was an increase in TGF-beta and miR23a in a 30% size graft. When SD208 (TGF-beta inhibitor) was injected, there was a decrease in both factors, with subsequent increase in mitochondrial activity. In addition, there was significant liver regeneration as opposed to suppression when the SD208 was administered. They concluded that TGF-beta inhibitors could potentially be used in the treatment of SFSG dysfunction.[8]

Gu et al.[9] using a mouse model of hepatotoxic drug-induced liver injury and analysing patient samples demonstrated that CHI3LI (a prototypic chitinase-like protein that has been retained over species and evolutionary time) protects livers against liver injury. Knockout of CHI3LI (CHI3LI-KO) resulted in exacerbating liver injury. The CHI3L1 levels were decreased in patients after liver injury. High levels of CHI3L1 are associated with the

improvement of liver function followed by medical treatment. CHI3LI-mediated hepato-protection by inhibiting Th17 and promoting Foxp3+Tregs in a Stat3-dependent manner. Their findings established the regulatory role of CHI3L1 in liver injury, and may provide novel therapeutic targets in liver injury and liver failure.

Bontha et al.[10] performed a detailed analysis pre implantation and post reperfusion allograft biopsies obtained from deceased donor kidney transplant recipients (n = 30) and liver transplant recipients (n = 30) using 120 gene expression (Gene Chips) arrays. They identified common intra-allograft inflammatory responses to IRI between kidney and liver transplantation. It was also observed that 40-50% of the differentially expressed genes were similar in both organs and these genes were mainly involved in inflammatory pathways and transcription regulation. Approximately 28% of the common genes were transcription factors and 12% were cytokines and growth factors. Among the top common pathways were IL-6 signalling pathway, NF-KB signalling, HMGB1 signalling, and production of NO and ROS in macrophages.[10] This study revealed common and specific pathways affected in IRI between two different transplanted organs.

These approaches may lead to the discovery of new therapeutic targets, improve common therapeutic interventions during IRI, and may help to identify organ specific pathways that can be specifically targeted to minimize injury. It was recently demonstrated that one molecular pathway responsible for the formation of nitric oxide by the endothelial cells is dependent on the stimulation of the glypican-1 (GP-1), a proteoglycan present in the Glycocalyx. Lopez et al.,[11] preserved Zucker rat fatty livers for 24hr in static cold storage in IGL-1 (n=5), UW (n=5) or HTK (n=5), and measured Glycocalyx proteoglycans, demonstrating they were better preserved in IGL-1 compared to HTK and UW. The

protective mechanisms of glypican-1 through the formation of nitric oxide in fatty livers may be due to its better preservation of the endothelial glycocalyx components during static cold storage. [11]

It is well established that bile production and composition correlates with transplant outcome. Kollmann et al.[12] investigated bile composition in a model of porcine liver transplantation as a marker of bile duct injury and function. The authors compared heart-beating donation (HBD) with 30 and 60min ischemia in DCD liver transplantation. Bile production during NMP was significantly higher in HBD vs. DCD liver transplantation. Prolonged warm ischemia was associated with increased bile CO_2 and bile glucose levels. In addition, bile cholesterol was lower during NMP in DCD grafts with prolonged warm ischemic injury.[12] Real time assessment of the bile composition during NMP might help determine algorithms for graft viability and help with decision-making over acceptance of marginal liver grafts.

Guo et al.[13] developed a new model of procurement graft preservation with NMP in pigs, called ischemia-free liver transplantation (IFLT). In this preservation method, there is no cold ischemia and the organ is continuously perfused and oxygenated from retrieval until the time of transplant. Ten pigs were subjected to IFLT (6-hour NMP) or conventional liver transplantation (CLT) (6-hour cold storage). The post transplant graft function was better with minimal histological changes after IFLT. There were fewer apoptotic hepatocytes, less sinusoidal endothelial cell injury and pro inflammatory cytokines (IL-1β, IL-6 and TNF-α) release.[13] The same group showed the first series of IFLT in 6 human transplants using IFLT with a 30-day graft survival of 100%.[14]

Matrix metalloproteinase-9 (MMP-9) facilitates leukocyte transmigration across vascular barriers in hepatic IRI. Tissue inhibitor of metalloproteinases-1 (TIMP-1), the endogenous MMP-9 regulator, is insufficiently expressed in liver to inhibit an elevated

MMP-9 activity post reperfusion. Duarte et al.[15] showed that gene therapy with viral vector (rAAV8-TIMP1 treated C57/BL6 mice) improved liver preservation histologically and led to significantly lower serum AST and ALT levels at 6hr and 24hr post-IRI. TIMP-1 gene transfer to TIMP1$^{-/-}$ mice restored TIMP-1 expression in liver, and enhanced the 7- day survival rate from 50% to 100% post-IRI. This study demonstrated that TIMP-1 acts as an endogenous hepatoprotective factor, and may be useful for treatment of clinical liver IRI.[15]

Organ Bioengineering / Bioprinting / Bioartificial liver

Bioprinting and biofabrification seek to produce tissue constructs from cells for tissue implants.[16,17] Li et al.[18] using a novel scaffold-free 3D bioprinting technology, sought to bioprint the liver using genetically-engineered porcine cells. Optimization of 3D-bioprinting was based on (i) porcine hepatocyte isolation; (ii) spheroid diameter, roundness, smoothness, durability, stability, and viability; and (iii) the ratio and combination of different cell types (fibroblasts, liver derived cells [LDC, CD31+], and/or hepatocytes). The investigators also developed a biocompatible, 3D-printed bioreactor capable of containing, culturing, perfusing, and observing biofabricated tissues aseptically in real-time. Using a combination of porcine fetal fibroblasts and LDCs, spheroids were generated after 2-3 days of plating and successfully used to bioprint three preliminary 3D liver constructs. The 3D-liver construct was perfused continuously for 1 week using this novel bioreactor. The authors therefore successfully bioprinted a scaffold-free 3D Bioprinted Porcine Liver Model, as a proof-of-concept for human liver bioprinting, and were able to continuously perfuse 3D-bioprinted livers for one week.[17,18]

The concept of using allo- or xeno-organ scaffolds to recreate organs with stem cells has the potential to increase the pool of organs and avoid organ rejection.[19] Willemse et al.[20] developed a protocol to complete remove of cellular debris from deceased pig livers using the pig liver matrix as a scaffold with further recellularization with human liver-derived

organoids (40 millions HepG2 cells or human-derived organoids). After the inoculation of the liver scaffolds and incubation, histological analysis was done and cells were found in the parenchymal areas of the liver segments showing that recellularization of porcine liver scaffold with human organoids is feasible with HepG2 cells.[20] Although the success was shown with HepG2 cells, the challenge will be to repeat the same experiment with primary liver hepatocytes.

Artificial extracorporeal liver supportive devices could support patients with potentially reversible liver failure as bridging therapy to recovery or transplantation.[21] However, randomized clinical trials have not demonstrated clear beneficial effects.[22] The spheroid reservoir bioartificial liver (SRBAL) is an experimental hepatic replacement therapy to achieve ammonia detoxification. Chen et al.[23] assessed SRBAL in post hepatectomy pigs. Pigs underwent intracranial pressure (ICP) monitor placement followed by 85% hepatectomy, confirmed by CT volumetric measurement. They were then randomized into standard therapy, bioartificial liver with 0gm of hepatocytes, and bioartificial liver with 200gm of hepatocyte spheroids. In the standard therapy group (0 gm), all animals had Grade IV hepatic encephalopathy or ICP >20 with herniation, while 5 out of 6 animals in the 200gm group survived up to 90 hours, with decreased ICP and INR, and evidence of liver regeneration on CT.[23]

Pig livers would represent an unlimited source of hepatocytes for both bioengineered livers and direct hepatocyte replacement. Nelson et al.[24] attempted to create a new model to study xenotransplantation of hepatocytes. Livers of fumarylacetacetate hydrolase (FAH) deficient pigs provide an advantage for the implantation of (FAH^+) donor hepatocytes. However, the host immune response against non autologous donor cells blocks this advantage. They hypothesized that the knockout of recombinase activating gene-2 (RAG2) will produce a severe combined immunodeficient (SCID) phenotype and may allow robust

expansion of non autologous hepatocytes. They produced thirteen FAH/RAG2 double-knockout piglets. They demonstrated variable growth curves from normal weight gain to moderate failure to thrive. The induction of a SCID phenotype may allow for consideration of xenotransplantation of hepatocytes in the future.[24]

Hepatocellular Carcinoma (HCC) Tumor Biology

It is suspected that HCC recurrence is more frequent after living donor than deceased donor liver transplantation[25] through SFSG providing a favourable immune microenvironment for tumor growth. Yang et al.[26] aimed to investigate the role of CCR5(+) NK cells in tumor recurrence after liver transplantation. They discovered that decreased peripheral activated NK cells resulted in increased risk of HCC recurrence among LDLT recipients. Inhibition of NK cell recruitment by blocking PD-L1 in $TLR4^{-/-}$ mice increased HCC recurrence. Therefore, the authors concluded that PD-L1/TLR4 signalling upregulated during liver graft injury directly induced the exhaustion of CCR5(+) NK cells, which further promoted HCC recurrence after transplantation.[26] This data has a great clinical relevance since targeting this pathway may decrease the incidence of HCC recurrence after LDLT. It is well known that the biology of the tumor represents a critical prognostic factor for HCC progression and recurrence post transplant.[27] Baciu et al.[28] aimed to determine a proteomic/transcriptomic signature of HCC recurrence in liver transplant patients with HCC within Milan criteria. Using high-throughput proteomics/transcriptomics and integrating the data, the authors found that 79 proteins were differentially expressed between recurrent and non recurrent cases. They also identified 3 proteins predictive of recurrence (ALDH1A1, Galectin-3 and Galectin-3 binding protein), independent of clinical risk factors, such as microvascular invasion, AFP and tumor size/number.[28]

Yeung et al.[29] showed that a subpopulation of macrophages (M2) has been associated with poor recurrence-free survival in HCC patients after hepatectomy. The authors analyzed M2 macrophages (expressed with a natural isoform of a key immune regulator) and its association with tumor recurrence in the context of living and deceased donor liver transplantation, and liver graft injury in a rat transplant model. A 2.1-fold increase of intra-graft M2 was found in liver grafts from living donors compared to deceased donors, and Δ42PD-1 was highly expressed in these cells associated with shorter recurrence-free survival in HCC recipients. In the rat transplant tumor model, liver injury was associated with accumulation of M2 macrophages and pro tumor cytokines secretion after transplantation and in larger tumor volume. They concluded that targeting this specific population represent a potential therapeutic strategy in attenuating HCC tumor recurrence.[29]

Although circulating microRNAs (miRNAs) play important roles in tumorigenesis and metastasis, they include miRNAs passively released from apoptotic and necrotic cells. Circulating exosomal miRNAs are more specific markers in tumorigenesis than circulating miRNAs. Lam et al.[30] quantified 754 exosomal miRNAs from pre operative serum of HCC patients. Of those, miRNA-1290 was found to have significant association with HCC recurrence and predicted the recurrence following tumor resection. High-level of miRNA-1290 was an independent predictor of poor overall survival and disease-free survival. Authors concluded that exosomal miRNA-1290 might serve as a prognostic biomarker for HCC recurrence and a potential therapeutic target.[30]

Li et al.[31] investigated the relationship between tumor stem cell marker epithelial cell adhesion molecule (EpCAM) in HCC tissue samples and HCC recurrence in patients undergoing liver transplantation. They showed that EpCAM expression, maximum tumor diameter, and microvascular invasion were significant predictors of survival and recurrence. EpCAM expression was also associated with tumor metastasis. Authors concluded that

EpCAM can be a predictor for HCC distant recurrence and long-term survival of patients with HCC after transplantation.[31]

Injury Biomarkers (OMICS)

Liver transplantation provides a complex biological system affected by unique donor and recipient biology, allo-graft injury, recipient response to injury, and the impact of immunosuppression.[32] In current 'multi-omics' era, complex systems are best studied through integration of genomics, transcriptomics, proteomics, metabolomics and epigenomics. Cho et al.[33] tested samples of 118 patients (n=236 biopsies paired pre implantation and post reperfusion biopsies, and paired plasma samples collected at pre implantation, on post operative day 1 and 2). They identified the characteristic expression of mRNA and miRNA profiles and associated it with donor quality and short term outcomes. Discovering biomarkers of injury pathways that reflect in donor quality and graft function may also lead to targeted therapeutic interventions to avoid or stop tissue injury.[33] Fernandez et al.,[34] using pre and post reperfusion liver biopsies, investigated if epigenetic modifications predispose graft sensitivity to injury severity of IRI. They found that methylome/transcriptome correlations in severely injured grafts were associated with apoptosis signalling activation, ubiquitin protein degradation, and cell cycle regulation. They identified 94 genes of liver damage, apoptosis and cell cycle regulation in severely injured grafts at post reperfusion biopsy.[34]

Wang et al.[35] compared circRNA sequencing of donor liver tissues between early allograft dysfunction (EAD) and non-EAD patients using Illumina Hiseq 3000 platform. A total of 431 circRNAs were identified differentially expressed between EAD and non-EAD donor liver tissues, of which 332 circRNAs were significantly up-regulated and 99 circRNAs were down-regulated. They showed that circRNA hosting genes were mainly involved in

metabolic process, protein binding and apoptosis, and certain circRNAs are potentially diagnostic biomarkers and therapeutic targets for EAD after liver transplantation.[35]

Bontha et al.[36] investigated the utility of cell-free DNA and mRNA panels post liver transplantation as biomarkers of severe IRI analysing biopsies and plasma of 64 DDLT and 10 LDLT. Cell-free circulating DNA corresponded to 95% to the liver graft at the time post reperfusion and decreased to 5% on POD2. Circulating levels of miRNA-16, -155, and -146a significantly increase in DDLTs, while miRNA-122 significantly decreased in LDLTs after 48hrs. In addition, cell-free circulating DNA was found to be elevated post reperfusion and was associated with the degree of injury.[36]

Pagano et al.[37] analysed the liver perfusate after whole graft washout in adult DBD with flow cytometry and the predicting role of NK cell subset on the biopsy-proven acute cellular rejection (ACR). The NK cell subset of liver perfusate was significantly associated with moderate-severe ACR and it was associated with any grade of rejection thus conceivably useful as a pre operative predictor of the risk of ACR.[37] Chruscinski et al.[38] reported a novel biomarker gene set for the identification of tolerance in murine transplant models. An 8-gene expression panel, which consisted of the increased expression of 6 immunoregulatory genes and decreased expression of 2 pro inflammatory genes, were found to be predictive of tolerance. In Phase 2A single centre clinical study (LITMUS) (n=54), they examined a panel of 8 target and 5 housekeeping gene expressions in the peripheral blood mononuclear cells (PBMC). Of the 10 remaining patients, 5 have been weaned off of immunosuppression, 2 are undergoing withdrawal and 3 developed ACR, which was easily reversed.[38] These data suggest that a combination of gene expression monitoring in PBMC and liver allograft may identify operationally tolerant recipients.

Miscellaneous (Transplant Immunology, Biliary Injury/Regeneration, Microbiomes)

Kubal et al.[39] showed that the development of de novo specific antibodies (dnDSA) has been identified as a risk factor for complications after liver transplantation. It is believed that HLA epitope/eplet mismatch is a potential risk factor for dnDSA formation. 80 liver transplant patients were analysed. 34% developed dnDSA and 11% developed ACR. Class II epitope/eplet mismatches were strongly associated with risk of Class II dnDSA formation and rejection after liver transplant and they concluded these patients may benefit from closer monitoring.[39]

The biliary tree can be damaged by ischemia during and after liver transplantation, in the setting of hepatic artery complications (especially in LDLT) or DCD liver transplantation. It has been previously shown that the biliary tree harbors stem cells that contribute to bile duct homeostasis and repair. Burka et al.[40] aimed to confirm the presence, expand and characterize biliary stem cells using 3D cultures of human bile duct organoids. They used human extrahepatic bile ducts (n=32) collected from donor or explant patient livers during liver transplantation. They were able to demonstrate the presence of LGR5-positive stem/progenitor cells, which can be expanded long-term in 3D cultures. In the future, these organoids can potentially be used for bile duct regeneration in damaged grafts prior to liver transplantation.[40] This would be particularly interesting to be tested during machine perfusion preservation of DCD grafts.

Visseren et al.[41] evaluated pre transplant colonic microbiome in 100 patients who underwent transplant for primary sclerosing cholangitis (PSC), by extracting bacterial DNA from samples of screening colonoscopy biopsies and performing 16S rRNA sequencing. They determined that those with recurrent PSC post transplant (14% of the total population) had significantly more abundant *Microbacterium (phylum Actinobacteria)* and *Thermicanus (phylum Firmicutes)*. Results suggest a potential contribution of these bacteria to the

pathogenesis of recurrent PSC.[41] Thus, modulation of the gut microbiome would have a critical role in preventing or alleviating liver disease. While a sophisticated understanding of the role of the microbiome in liver transplantation is in its infancy, the potential deserves both basic and clinical research.[42]

New targets to understand liver fibrosis are of key importance to prevent the development of cirrhosis. Liver fibrosis frequently results from chronic damage to the liver, which leads to accumulation of extracellular matrix proteins. microRNAs (miRNAs) are reported to play a critical role in the development of liver fibrosis. Hu et al.[43] suggested that lower miR-152 expression might be involved in generation of liver fibrosis by promoting Gli3 expression, and overexpression miR-152 could reduce the process of liver fibrosis. These findings will provide insight into miR-152 potential as an anti-fibrotic therapy through modulating Gli3.[43]

Hessheimer et al.[44] investigated the impact of changes in gene expression on coagulopathy in high-risk uncontrolled DCD (uDCD) liver transplantation using microarrays and RT-PCR. uDCD livers had significantly upregulated expression of genes that provoke fibrinolysis and inhibit coagulation, including TPA, urokinase plasminogen activator, urokinase plasminogen activator receptor, and thrombomodulin. There was also decreased expression of genes implicated in hemostasis von Willebrand factor gene and inhibition of fibrinolysis (alpha-2-macroglobulin). Interestingly, no fibrin microthrombi were detected, even in the patients who developed ischemic-type biliary lesions. Endogenous fibrinolysis is upregulated in uDCD livers at risk for ischemic cholangiopathy, and clinical DCD protocols using TPA to help prevent ischemic cholangiopathy should undergo critical appraisal.[44]

Liver Xenotransplantation

With the development of the gene-editing technology CRISPR/Cas 9, there is a renewed interest in liver xenotransplantation.[45] It is known that platelet sequestration and coagulation cascade activation are associated with poor outcomes in multiple liver xenotransplant models. Cimeno et al.[46] ex vivo perfused livers from α1,3-galactosyl transferase knockout (GalTKO), and human membrane cofactor (hCD46) pigs (Group 1, n=3) and GalTKO.hCD46 pigs also transgenic for human endothelial protein C receptor (hEPCR), thrombomodulin (hTBM), integrin associated protein (hCD47), and heme oxygenase 1 (HO-1) treated with DDAVP and clodronate liposomes (Group 2, n=4) with whole human blood. Transgenic expression of the hEPCR.hTBM.hCD47.HO-1 cassette, along with donor pig DDAVP and clodronate liposome pretreatment, was associated with improved liver xenograft survival in ex vivo perfusion model.[46]

Xenograft vascular endothelium represents the initial site of recipient immune exposure to xenoantigens. Hassenein et al.[47] successfully engrafted human umbilical vein endothelial cells (HUVECs) into the de endothelialized rat livers. This method demonstrates the ability to manipulate a key component of the immune response to xenogeneic antigen and biologically engineered liver xenografts after re-endothelialization of the vascular tree with allogenic endothelial cells has the potential to evade the recipient´s immune response.[47]

Conclusions

One of the substantial tasks for ILTS-ELITA-LICAGE is to nurture and maintain vibrant basic and translational science in liver transplantation. The 2018 meeting has demonstrated that advances including gene-editing, the various 'omics' technologies, bioengineering, bioprinting and new approaches to organ preservation and revitalization are yielding progress. The field needs to research long-term goals, such as identifying patients who will most benefit from a transplant and through expanding the potential donor pool of organs for

liver transplantation using ex vivo perfusion, xenotransplantation, organ and tissue engineering and bioprinting. Basic science discoveries have yielded several areas that may help to transform the field of liver transplantation over the next decade.

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Figure 1: Distribution of delegates to the 2018 Joint ILTS-ELITA-LICAGE Meeting

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Figure 1.